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Lentiviral vectors carrying enhancer elements of Hb9 promoter drive selective transgene expression in mouse spinal cord motor neurons

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ABSTRACT

Recombinant lentiviral vectors (rLVs) have emerged as versatile tools for gene delivery applications due to a number of favorable features, such as the possibility to maintain long-term transgene expression, the flexibility in the design of the expression cassettes and recent improvements in their biosafety profile. Since rLVs are able to infect multiple cell types including post-mitotic cells such as neurons and skeletal muscle cells, several studies have been exploring their application for the study and cure of neurodegenerative diseases. In particular, the introduction of rLVs carrying cell-type specific promoters could restrict the transgene expression either to neuronal or glial cells, thus helping to better dissect *in vivo* the role played by these cell populations in several neurodegenerative processes. In this study we developed rLVs carrying motor neuron specific regulatory sequences derived from the promoter of homeobox gene Hb9, and demonstrated that these constructs can represent a suitable platform for selective gene-targeting of murine spinal cord motor neurons, *in vivo*. This tool could be instrumental in the dissection of the molecular mechanisms involved in the selective degeneration of motor neurons occurring in Motor Neuron Diseases.

1. Introduction

Dysfunction and degeneration of motor neurons are the key features of a group of pathologies with high social impact termed Motor Neuron Diseases (MND), which include fatal syndromes such as Amyotrophic Lateral Sclerosis (ALS) (de Carvalho and Swash, 2011). The possibility to visualize motor neurons and their neurites, or to manipulate at a molecular level the pathways that are modified in these cells in pathological conditions, has catalyzed great interest. In fact, this approach would help to dissect, in deeper detail, the molecular mechanisms involved in motor neuronal functions in physiologic or stressful conditions, such as in MND. Despite recent advances obtained through transgenic mice carrying cell-type specific expression cassettes (Ezzi et al., 2010; Jaarsma et al., 2008; Reyes et al., 2010; Shneider et al., 2009), post-natal gene delivery *in vivo* appears more attractive, since this approach would aid expression of the transfected genes in tissue-specific and time-modulated manner, with possible implications for gene-therapy in humans. It has been demonstrated that recombinant lentiviral vectors (rLVs) can easily transduce slowly- and non-dividing cells (like neurons), thus they are considered powerful tools for gene transfer to the central nervous system (CNS) and hold great potential as a therapeutic gene delivery strategy for neurological disorders. rLVs appear particularly suitable for applications in chronic pathologies, since they can drive long-lasting expression in the brain due to the fact that they integrate in the host genome (Coffin et al., 1997). Though this aspect can rise safety concerns, mainly because of the risk of insertional mutagenesis, integration-deficient rLVs can now be used to overcome this problem (Kumar and Woon-Khiong, 2011; Matrai et al., 2010). Moreover, several improvements have been recently introduced in viral vector design to increase infection efficiency and the safety profile (Kumar and Woon-Khiong, 2011; Matrai et al., 2010). It has already been shown that injection of the rLVs into the brain parenchyma, through stereotaxic surgery, leads to transduction of brain areas such as the striatum,

Abbreviations: ALS, Amyotrophic Lateral Sclerosis; ChAT, choline acetyl transferase; CNPase, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; EF1a, elongation factor 1 alpha; GFAP, Glial Fibrillary Acidic Protein; MAP2, microtubule-associated protein 2; MBP, Myelin Basic Protein; MND, Motor Neuron Diseases; NF-H, neurofilament heavy chain; rLVs, recombinant lentiviral vectors; NK-10, zinc-finger protein 90; NSE, Neuron Specific Enolase; REST, RE1-silencing transcription factor; VSV-G, Vesicular Stomatitis Virus Glycoprotein.

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hippocampus and thalamus (Watson et al., 2002), without relevant damages to the injected tissue, with the exception of a mild and transient inflammatory reaction in the area immediately surrounding the injection site (Abordo-Adesida et al., 2005). A number of studies have described the use of this technique for delivery in the spinal cord of rats (Abdellatif et al., 2006; Hendriks et al., 2007; Zhao et al., 2003) and mice (Guillot et al., 2004). In the latter case, Guillot and colleagues demonstrated that delivery of beta-Gal-expressing lentivector in the spinal cord of 40-days-old mice leads to robust reporter gene expression in neurons, including motor neurons, and in glial cells, with the signal widely distributed in the spinal cord parenchyma, and an absence of deterioration of motor behavior in the animals. The rLVs used in this work were pseudotyped with Vescicular Stomatitis Virus Glycoprotein (VSV-G), which gives broad tropism, and carried a transgene under a ubiquitous promoter. Regulatory sequences of rat Neuron Specific Enolase (rNSE), human Glial Fibrillary Acidic Protein (hGFAP) and Myelin Basic Protein (MBP) have already been exploited to obtain rLV-mediated selective gene-targeting of neurons, astrocytes and oligodendrocytes, respectively (Jakobsson et al., 2003; McIver et al., 2005). However, up to date tests of regulatory sequences able to drive rLV-mediated gene expression in motor neurons are lacking.

In this work we decided to develop rLVs carrying motor neuron-specific regulatory sequences to obtain transcriptional targeting of spinal cord motor neurons *in vivo*, as potential tool for studies on animal models of MND, and as a platform for generation of future more specific gene-therapy approaches.

2. Materials and methods

2.1. Plasmid constructs

A plasmid containing the rat Neuron Specific Enolase promoter (LV.rNSE) was kindly provided by Dr Cecilia Lundberg (Lund University, Sweden). The ClaI/BamHI fragment derived from LV.rNSE, containing the rNSE promoter elements, was subcloned into the ClaI/SwaI site of pWPXLd lentiviral vector (Addgene Plasmid 12257), BamHI and SwaI sites destroyed, thus generating the WPX.NSE.GFP vector. The 9 kb 5′-flanking region of mouse Hb9 gene (Hb9 promoter) was a kind gift of Dr Thomas Jessell (Columbia University, USA). Construct Bg.Hb9_3.6 was generated by inserting a NotI/SbfI fragment (3.6 kb) derived from the Hb9 promoter into NotI/BamHI sites (SbfI and BamHI sites destroyed) of Bg.GFP, which contains beta-globin minimal promoter and GFP as reporter gene (kind gift of Dr. Jane Johnson, UT Southwestern Medical Center, USA). Construct Bg.Hb9_1.6 was obtained by insertion of a NotI/NdeI fragment (1.6 kb) derived from the Hb9 promoter into Bg.GFP. Bg.Hb9_AB construct was generated as follows: a 313 bp fragment (construct A) and a 125 bp fragment (construct B) were PCR amplified from the Hb9 promoter using separated sets of primers (5′-ATAGCATAGCGGCCGCTGAATAAATTTAAGCAGGCT-3′, 5′-GCTCTAGAAGCCCCATCCCCCTTCAAT-3′ for construct A and 5′-GACTAGTAGAGTGGTTAGCTGATGAAT-3′, 5′-TCACCCGGGTCTAA-TCAGCCTGCCTAGCT-3′, for construct B). Construct A and construct B were then fused together, sequence verified and cloned inside NotI/SmaI sites of Bg.GFP. For generation of W.Hb9_1.6 and W.Hb9_AB viral vector constructs, the promoter sequences were excised by SalI/SbfI digestion from Bg.Hb9_1.6 and Bg.Hb9_AB, respectively, and then inserted into SalI/SwaI sites of pWPXLd, after blunting of SbfI.

2.2. Viral vector production

Production of high titre lentiviral vectors was performed by trasfection of 293T cells with 4 plasmids: pRSV-Rev and pCMVdR8.74 (provided by Trono Lab, Lausanne, Switzerland) encoding gag/pol/rev proteins, pWPXLd or constructs derived from pWPXLd containing different promoters, and pMD2G (BD Biosciences) for expression of Vescicular Stomatitis Virus G glycoprotein (VSV-G).

Briefly, 293T cells were plated at 70% confluence in Dulbecco's Modified Medium, 10% FCS, 1% glutamine, 1% penicillin, 1% streptomycin and maintained at 37 °C in a humidified 5% CO_2 incubator. Co-transfection of the four plasmids was performed with Effectene kit (Quiagen) according to the manufacturer instructions. After 16 h the medium was replaced by fresh culture-medium containing 10 mM sodium butyrate (Sigma). Supernatants were harvested 24 h and 48 h post-transfection, filtered through a 0.45 micron filter and stored at 4 °C. New medium with 10 mM sodium butyrate was added to the cells after the first harvesting. Concentrated viral stocks were produced by ultracentrifugation at 50,000 × *g* at 4 °C for 2 h. Viral pellets were resuspended in sterile PBS and then stored at −80 °C till the experiment. The viral titre of the viral stocks was in the range of 1.0–2.0 × 10^9 T.U./mL, as assessed by end-point dilution assay performed on HEK293 cell line (Blesch, 2004). The titre of viral vectors carrying neuron specific promoters was assayed on neuronal cultures.

2.3. Surgery

C57BL/6 female mice (Harlan Italy) at 10–12 weeks of age were used in the experiments. Thirty minute before the surgery, animals received an injection of ampicillin (0.03 mg/kg, s.c.) and buprenorphine (0.05 mg/kg, s.c.). All surgical procedures were then performed under deep anesthesia by 2.5% Avertin (2,2,2 tribromoethanol in 2 methyl-2-butanol, 400 mg/kg, i.p.). Animals' back was shaved at dorsal level, and a cutaneous incision (3 cm) was performed to expose the backbone. T_{13} and L_1 vertebrae were identified and exposed by separation of dorsal and intervertebral muscles. Animals were then placed on a Cunningham Spinal Cord Adaptor (Stoelting) mounted on a stereotaxic frame, and laminectomy of L_1 vertebra was performed to uncover lumbar spinal cord at levels L_2–L_4. Using a glass capillary (40 ± 5 μm diameter) viral solution was injected in the spinal cord in two sites separated 2 mm along the spinal cord (1.5 μl/site; flow rate of 0.2 μl/min). Stereotaxic coordinates were referred to the midline of the dorsal horn of spinal cord; the needle was positioned at ±0.5 mm aside from the midline, then it was deepened into the parenchyma to 0.8 mm below the pia mater to reach the ventral horn where motor neurons are located; the injector was left in place for 1 min and then retracted for 0.2 mm before starting the delivery. After completion of the injection the needle was left in place for additional 2 min and then gently withdrawn. Dorsal muscles were then juxtaposed by absorbable sutures, and skin sutured and disinfected. After surgery animals were kept on a warm pad for 30 min and then placed in separated cages for recovery. Two weeks after surgery mice were deeply anesthetized and transcardially perfused with 50 mL of phosphate buffered saline (PBS, phosphate buffer 0.01 M, 0.9% NaCl) followed by 50 mL of 4% paraformaldehyde solution in PBS. Lumbar spinal cord segments were rapidly removed, post-fixed in fixative for 3 h, transferred to 20% sucrose solution in PBS overnight, then to 30% sucrose solution until they sank, and finally frozen in 2-methylbutane at −45 °C and conserved at −80 °C until the experiments.

Procedures involving animals and their care were conducted in according to the institutional guidelines, that are in compliance with national (D.L. no. 116, G.U. suppl. 40, Feb. 18, 1992, Circolare No. 8, G.U., 14 luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1 DEC.12, 1987; NIH guide for the Care and use of Laboratory Animals, U.S. National Research Council, 1996). The animals were housed under standard conditions (22 ± 1 °C, 60% relative humidity, 12 h light/dark schedule),